

**LOW DHEAS: A SENSITIVE AND SPECIFIC TEST FOR THE DETECTION OF
SUBCLINICAL HYPERCORTISOLISM IN ADRENAL INCIDENTALOMAS.**

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Abbreviated title: DHEAS and subclinical hypercortisolism in AI.

Key words: DHEAS, subclinical hypercortisolism, adrenal incidentaloma

Word Count: Abstract 249; Text 3580; Figures 3; Table 1

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Disclosures: The authors have nothing to disclose.

Abstract

Context: Subclinical hypercortisolism (SH) occurs in 5-30% of adrenal incidentalomas (AIs). Common screening tests for adrenocorticotropin (ACTH)-independent hypercortisolism have significant false positive rates, mandating further time and resource intensive investigations.

Objective: To determine whether low basal dehydroepiandrosterone sulphate (DHEAS) is a sensitive and specific screening test for SH in AI.

Setting and Patients: 185 patients with AI were screened for adrenal medullary (plasma metanephrines) and cortical [1 mg overnight dexamethasone suppression test (ONDST), 24h urinary free cortisol (UFC), serum DHEAS, plasma renin and aldosterone] hyperfunction. Positive ONDST [≥ 1.8 mcg/dL (≥ 50 nmol/L)] and/or UFC (>upper limit of reference range) results were further investigated. We diagnosed SH when at least two of the following were met: raised UFC, raised midnight serum cortisol, 48h DST cortisol ≥ 1.8 mcg/dL (≥ 50 nmol/L).

Results: Twenty-nine patients (16%) were diagnosed with SH. ACTH was <10 pg/mL (<2.2 pmol/L) in all patients with SH. We calculated age- and gender-specific DHEAS ratios (derived by dividing the DHEAS by the lower limit of the respective reference range) for all patients. ROC analyses, demonstrated that a ratio of 1.12 was sensitive ($>99\%$) and specific (91.9%) for the diagnosis of SH. Cortisol following 1mg ONDST of 1.9 mcg/dL (53 nmol/L) was a sensitive ($>99\%$) screening test for SH, but had lower specificity (82.9%). 24 h UFC lacked sensitivity (69%) and specificity (72%).

Conclusion: A single basal measurement of DHEAS offers comparable sensitivity and greater specificity to the existing gold-standard 1 mg DST for the detection of SH in patients with AIs.

Introduction

Incidentally discovered adrenal lesions [so-called adrenal incidentalomas (AIs)] are a common cause of endocrine referral (1). Although most are benign and not associated with overt endocrine dysfunction, subtler forms of adrenal hypersecretion (hypercortisolism, hyperaldosteronism, sex-steroid excess or pheochromocytoma) have been variably reported in up to 20–40 % of AIs (2,3). The terms subacute autonomous glucocorticoid hypersecretion (SAGH), subclinical Cushing's syndrome (SCS), (possible) autonomous cortisol secretion, and subclinical hypercortisolism (SH) are often interchangeably used to denote adrenocorticotropin (ACTH)-independent cortisol secretion from a benign adrenal adenoma or nodular adrenal hyperplasia that is not associated with clinically-overt hypercortisolism (2,4,5). Several groups have reported adverse clinical sequelae in individuals with SH, with recent studies highlighting an increase in cardiovascular morbidity and mortality compared to the general population (6-12). While independent medical treatment of cardiovascular risk factors may represent a reasonable long-term management strategy for individuals with SH, recent advances in laparoscopic and retroperitoneoscopic adrenalectomy raise the possibility of a potentially curative intervention for a subgroup of patients (13,14). A related, but frequently under-appreciated risk in these patients is chronic hypothalamic-pituitary-adrenal axis suppression, resulting in an impaired or absent stress response to intercurrent illness (7). Accurate exclusion or confirmation of a diagnosis of SH is therefore a key step in the investigation and management of patients with AIs.

The 1 mg (overnight) dexamethasone suppression test (ONDST) is often employed as a sensitive screening test to exclude SH (1,4,15), but its relatively poor specificity (70–80%) results in a significant number of false positive tests, requiring further evaluation that is both time and resource intensive (4). This has led some workers to propose a higher threshold [$\geq 5\text{mcg/dL}$ ($\geq 138\text{ nmol/L}$)] for triggering additional screening/confirmatory investigations in the context of AI (15), but with the inevitable consequence of failing to diagnose milder cases of SH. Measurement of 24 h urinary free cortisol excretion rates (UFCs) is also potentially problematic with causes of both false positive (e.g. obesity) and false negative (e.g. mild hypercortisolism, renal impairment) results recognised (4,16). Demonstration of the loss of the normal circadian rhythm of cortisol secretion is considered a sensitive

test for the detection of hypercortisolism, but opinions vary as to the thresholds that should be employed to optimize detection while minimizing false positive results (for example, although a sleeping midnight serum cortisol of <1.8 mcg/dL (<50 nmol/L) effectively excludes Cushing's syndrome in the absence of cyclical hypercortisolism, false positive results are common, especially in patients hospitalized for short periods (<48h) (4,17-19). Similarly, late night salivary cortisol is sensitive for the detection of hypercortisolism, but may be falsely elevated in shift workers, smokers or if there is blood contamination of the sample (4,20,21). Early morning (9 AM) plasma ACTH levels are typically low or suppressed in SH, but there is overlap with ACTH levels seen in normal individuals, reflecting its pulsatile secretion and short half-life (4,22).

Synthesis and secretion of the adrenal androgen dehydroepiandrosterone (DHEA), and its sulphated form DHEAS, are regulated by pituitary ACTH. Sustained suppression of central ACTH drive therefore leads to a reduction in DHEA and DHEAS levels (23-25). However, DHEA has a short half-life (25 mins) and is secreted in a circadian manner similar to ACTH; hence, the interpretation of a single DHEA measurement is subject to the same caveats as with ACTH. In contrast, DHEAS has a prolonged half-life in serum (10–16h), with relatively stable levels throughout the day, making it a more attractive marker for the detection of chronically suppressed ACTH. (26,27). Indeed, the potential utility of DHEAS to reflect ambient ACTH levels over a longer time interval has led to DHEAS being proposed as an indicator of persisting autonomous ACTH secretion in Cushing's disease following pituitary surgery (28), and as a possible marker of SH in patients with AI (29, 30, 31).

We therefore performed a systematic comparison of a single DHEAS measurement at presentation with the current gold-standard 1 mg dexamethasone suppression test, for the detection of SH in 185 consecutive individuals with AI referred to the endocrine department of a university hospital. We describe the utility of DHEAS as a sensitive and specific marker of SH within this cohort.

Patients and Methodology

Patients

The records of 185 consecutive patients with adrenal incidentalomas referred to the Endocrine Department of Cambridge University Hospitals Foundation Trust between January 2006 and April 2013 were reviewed. All patients had undergone standardized clinical, biochemical and radiological assessments according to an Institutional protocol, as part of routine clinical care. We performed a retrospective analysis of this systematically collected dataset. Appropriate Institutional approval was obtained.

Seventeen patients were excluded on the basis of: (i) concomitant use of drugs influencing glucocorticoid metabolism or secretion; (ii) major psychiatric illness or history of excess alcohol intake; or (iii) overt clinical features of hypercortisolism. One patient had previously undergone pituitary surgery and was also excluded. All AIs were discovered by computed tomography (CT) scanning or magnetic resonance imaging (MRI) performed for unrelated reasons, and all lesions were >1cm in diameter. Adrenal adenomas displayed the following radiological features: either ≤ 10 HU on unenhanced CT (in which case no further characterization was performed); or >50% absolute washout at 10 minutes (or >60% at 15 minutes) on triple phase adrenal CT; or signal dropout on chemical shift MRI (32,33).

All patients were assessed by one of three clinicians experienced in the diagnosis and management of Cushing's syndrome, and each underwent standardized clinical and biochemical screening at first presentation according to an institutional clinical protocol. This included measurement of plasma metanephrines, 24 h urine collection for urinary free cortisol estimation (UFC), plasma renin and aldosterone, serum DHEAS, electrolytes, liver blood tests, fasting plasma glucose, fasting lipids and complete blood count. All patients also underwent a 1 mg overnight dexamethasone suppression test, with values < 1.8 mcg/dL (< 50 nmol/L) signifying adequate suppression (34). Measurement of UFC was performed separately on two 24 h urine collections, with the higher of the two values used for subsequent analysis. Patients returning a post-dexamethasone cortisol ≥ 1.8 mcg/dL (≥ 50 nmol/L)

and/or raised UFC [>6.5 mcg/dL (>180 nmol)/24 h prior to 2011 and >5.3 mcg/dL (>146 nmol)/24 h after 2011] underwent more detailed evaluation during an inpatient admission as follows: repeat 24 h UFC, midnight serum cortisol, 48 h low dose (0.5 mg qds) dexamethasone suppression test (LDDST), plasma ACTH (measured on 2 occasions between 08:00 and 09:00). We diagnosed SH in the presence of at least two of the following: failure to suppress serum cortisol to <1.8 mcg/dL (<50 nmol/L) following dexamethasone; sleeping midnight serum cortisol >1.8 mcg/dL (>50 nmol/L) or awake midnight serum cortisol >7.5 mcg/dL (>207 nmol/L); raised UFC (32). Although not used as a diagnostic criterion, all patients subsequently confirmed with SH had a 09:00 ACTH level <10 pg/mL (<2.2 pmol/L). All investigations were reviewed by two endocrinologists (MCD, MG), who were blinded to the DHEAS result for each patient. For the purpose of analyses, a DHEAS ratio was calculated by dividing the measured DHEAS by the lower limit of the respective reference range (age- and sex-matched). DHEAS values reported as falling below the lower limit of detection of the assay [<15.0 mcg/dL (<0.4 micromol/L)] were interpreted as equivalent to the lowest measurable value [i.e. 15.0 mcg/dL (0.4 micromol/L)].

Assays

Biochemical assays were run in a UKAS (<http://www.ukas.com/default.asp>) accredited clinical laboratory. External Quality Assurance (EQA) for all assays was provided by UKNEQAS (<http://www.ukneqas.org.uk>).

DHEAS and ACTH were assayed using a Siemens (Surrey, UK) Immulite 2000 platform with reagents provided by the same manufacturer. DHEAS was analyzed by solid phase competitive immunoassay. Age and sex adjusted reference ranges for DHEAS are provided in Table 1. ACTH was analysed by sequential two site solid phase chemiluminescent immunoassay.

Aldosterone was measured using solid phase radioimmunoassay (Coat-A-Count, Siemens, Los Angeles, USA). The Laboratory working range was 70–3330 pmol/L. Plasma Renin was measured using a Diasorin XL (Kent, UK) chemilluminescent immunometric assay. The Laboratory working range was 2–3000 μ IU/mL.

Plasma metanephrines were analyzed using a derivitization ELISA assay provided by Labor Diagnosticka Nord GmbH (Nordhorn, Germany). The Laboratory working range was 41-2100 pg/mL for metanephrine and 58-58000 pg/mL for normetanephrine.

Urine cortisol was measured by competitive radioimmunoassay using reagents provided by Siemens (Coat-a-Count) or using an in-house liquid chromatography – tandem mass spectrometry method. The Laboratory working range was 25-1380 nmol/L and the laboratory reference range for the RIA was <180 nmol/24 h. The mass spectrometric assay was performed using an API 5500 triple quadrupole mass spectrometer (ABSciex, UK) using atmospheric pressure chemical ionization. Deuterated (d4) cortisol was used as an internal standard. Urine samples were mixed with internal standard in 50% methanol/water. Samples were pre-cleaned using a C8 reverse phase column before HPLC separation using a pheny-hex column with a shimadzu HPLC system. The Laboratory working range was 12–2330 nmol/L and the laboratory reference interval for the liquid chromatography–tandem mass spectrometry method was <146 nmol/24 h.

Between batch imprecision was <10% across the working ranges for all assays.

Statistical Analysis

With the exception of age and body mass index, which are reported as mean \pm standard deviation (SD), all data are expressed as medians with interquartile ranges. For parametric data, hypothesis testing was performed using an unpaired students t-test. For non-parametric data, statistical analysis was performed using the *Mann Whitney U Test*.

Receiver operated characteristic (ROC) curves were generated to assess the utility of DHEAS in the diagnosis of SH, using the first, randomly sampled DHEAS ratio for each patient included in the study. ROC curves were also generated for the 1 mg overnight dexamethasone suppression test and the original 24 h UFC measurement.

ROC analysis evaluated the performance of each parameter for the diagnosis of SH against non-functioning adrenal adenomas (NFAs). Performance of each screening / diagnostic test is expressed as

170 area under the curve (AUC) with 95% confidence intervals. Data are also presented for sensitivity and
171 specificity for each screening / diagnostic test with 95% confidence intervals. All analyses were
172 performed using SPSS V 21.0 or GraphPad Prism V 6.0.

173 Due to the systematic nature of investigation for AI in our Institution, and robust data collection, there
174 were no missing data for ONDST, LDDST, UFC or demographic details in the study cohort.

Results

Patient Characteristics

As described above, 18 subjects were excluded from further analyses due to confounding clinical disorders or medication usage. Final diagnoses in the remaining 167 patients were: non-functioning adrenal adenoma (n=97), subclinical hypercortisolism (n=29), phaeochromocytoma (n=19), adrenal metastasis (n=8), primary aldosteronism (n=4), adrenal hemorrhage (n=2), adrenocortical carcinoma (n=2), myelolipoma (n=2), oncocytoma (n=1) and three subjects had Cushing's disease with an adrenal incidentaloma (Supplemental Fig. 1A). There was no difference in age [NFA: 63.4 ± 12.8 yr (mean \pm SD); SH: 65.8 ± 11.3 yr ($p=0.36$)], sex [NFA: Male 44/97; SH: Male 14/29 ($p=0.41$)] or body mass index [NFA: 31.8 ± 9.2 kg/m² (mean \pm SD); SH: 29.4 ± 7.5 kg/m² ($p=0.91$)] between the NFA and SH subgroups. The age distribution of the cohort as a whole was consistent with other published series (Supplemental Fig. 1B) (6-11). Twenty-three patients had bilateral adrenal adenomas (16 NFA; 7 SH).

DHEAS ratio for the screening and diagnosis of SH

There was a significant difference in the DHEAS ratio between the two study groups [median 2.62 (1.8, 5.5) for NFAs versus 1.0 (0.77, 1.0) for SH; $p < 0.0001$]. DHEAS ratio was a sensitive and specific test both with respect to screening and diagnosis of SH versus NFA [AUC 0.95 (0.91, 0.99); $p < 0.0001$]. Evaluation for SH versus NFA showed that for a DHEAS ratio of 1.12, sensitivity was 100% (86.2, 100) and specificity was 91.9% (83.9, 96.7) (Fig. 1). This finding was consistent in a subgroup analysis of patients with bilateral adrenal adenomas (Supplemental Fig. 2A). In fact, DHEAS ratio also performed well as a sensitive and a specific test for the diagnosis of SH against all other causes of adrenal lesions within this cohort, as all other non-SH causes of adrenal nodules returned a DHEAS ratio > 1.12 . Included in this, a low DHEAS ratio also differentiated between ACTH-independent and ACTH-dependent hypercortisolemia in the presence of an adrenal lesion (Supplemental Fig. 3).

1 mg dexamethasone suppression test for the screening and diagnosis of SH

There was a significant difference between the two study groups with respect to post-1mg dexamethasone cortisol levels: median 1.3 mcg/dL (0.91, 1.71) [36 nmol/L (25.0, 47.3)] for NFAs, versus 4.2 mcg/dL (1.96, 8.73) [116 nmol/L (92.0, 241.0)] for SH ($p < 0.0001$). As expected, the 1 mg overnight dexamethasone suppression test performed well as a screening test for SH when compared with NFAs [AUC 0.97 (0.95, 0.99); $p < 0.0001$] (Fig. 2). A post-dexamethasone value of 1.9 mcg/dL (53 nmol/L) yielded a sensitivity $>99\%$ (87.2, 100) and a specificity of 82.9% (73.4, 90.1). As a diagnostic test, a value of 4.0 mcg/dL (109 nmol/L) had a sensitivity of 55.5% (35.3, 74.5) with a specificity of $>99\%$ (95.9, 100). A post-dexamethasone cortisol of 2.1 mcg/dL (58.5 nmol/L) gave the best overall test performance: sensitivity 92.5% (75.7, 99.0); specificity 88.6% (80.1, 94.4). Findings in those with bilateral adrenal adenomas were largely consistent with this (Supplemental Fig. 2B). The 1 mg overnight dexamethasone suppression test did not differentiate between SH and other causes of hypercortisolism such as ACTH-mediated or adrenocortical carcinoma.

24 hour UFC for the screening and diagnosis of SH

For the purpose of analyses, a UFC ratio was calculated by dividing the measured 24 hour UFC by the upper limit of the reference range. There was a significant difference in the UFC ratio between the two study groups: median 0.74 (0.46, 1.16) for NFAs versus 1.3 (0.95, 1.84) for SH ($p < 0.0001$) (Fig. 3). However, UFC performed relatively poorly both as a screening test and as a diagnostic test for SH versus NFA [AUC 0.77 (0.67, 0.87); $p < 0.01$], and this pattern held true for bilateral adenomas (Supplemental Fig 2C). A ratio of 1.01 gave a sensitivity of 69 % (48.2, 85.7) and a specificity of 67% (55.5, 78.2). A ratio of 0.47 gave a sensitivity of 100% (86.8, 100) but a specificity of 25.3% (15.8, 37.1), while a ratio of 2.1 gave a specificity of 100% (94.9, 100), but a sensitivity of just 19.2% (6.6, 39.4). Therefore, while UFC did not perform well as a screening test, a value greater than twice the upper limit of the reference range performed well as a confirmatory test (Fig. 3).

Discussion

In this study we have robustly evaluated the diagnostic utility of a single DHEAS measurement as a sensitive and specific marker of SH in adrenal incidentalomas, using a complete and systematically collected dataset. Consecutive patients referred to our service were investigated using widely accepted screening and confirmatory tests (4). For every patient we also calculated a ratio of measured DHEAS to the age- and sex-specific lower reference interval. Using this approach, DHEAS ratio performed well as a screening test for SH, with a threshold of ≤ 1.12 demonstrating a sensitivity $>99\%$ while showing higher specificity than the ONDST (91% versus 86% respectively) (Figs. 1 & 2). Moreover, DHEAS ratio reliably differentiated between SH and non-SH etiologies of adrenal nodules within our cohort (Supplemental Fig. 3).

Our findings lend important support to previous proposals and guidelines, which have observed that a low or suppressed DHEAS level should be considered a potential indicator of SH in the context of AI (29-31). Yener and colleagues have also explored the association between low DHEAS levels and SH, noting that an age-unadjusted DHEAS threshold of 40.0 mcg/dL provided a sensitivity of 68% and specificity of 75% for the diagnosis of SH (29). In the study reported here, we have demonstrated a higher sensitivity and specificity for low DHEAS in the diagnosis of SH. This may be explained by some important differences in study design. Firstly, rather than adopting a higher ONDST threshold [3.0 mcg/dL (83 nmol/L)] as a confirmatory test for SH, we have used the diagnostic criteria recommended in the Endocrine Society Clinical Practice Guidelines (4), which include the LDDST as a ‘gold standard’ confirmatory test. In our hands, when compared with the LDDST, the higher ONDST threshold of 3.0 mcg/dL (83 nmol/L) performed less well as a confirmatory test for SH, returning a sensitivity of 85% and specificity of 95%. Secondly, we have accounted for the expected age-related decline and sex-related differences in DHEAS by calculating a sex- and age-adjusted DHEAS ratio based on locally validated reference ranges, rather than using absolute DHEAS values (Table 1). Although a previous report by Bencsik and colleagues suggested that DHEAS may not be a useful indicator of hormonal activity in AI (34), the main focus of this study was to explore the potential for DHEAS to discriminate benign from malignant AI. Moreover, the analysis was restricted to only a

small number of histologically-confirmed cases (thus introducing potential selection bias). Importantly the authors noted that DHEAS measurement may have a role once the cortical origin and benign feature of the tumor has been confirmed.

In this study we employed robust criteria for the diagnosis of SH across multiple testing modalities, which allowed us to also examine the performance of investigations that are commonly used to detect/exclude hypercortisolism. The ONDST performed well as a screening test for SH. The traditional cortisol threshold of ≥ 1.8 mcg/dL (≥ 50 nmol/L) yielded a sensitivity $>99\%$ and a specificity of 81%, consistent with previous findings for this test (Fig. 2) (4). The higher suggested cut-off value for cortisol of ≥ 5 mcg/dL (>138 nmol/L) post-dexamethasone provided a specific test ($>99\%$), but at the cost of a low sensitivity of 41% which would have missed the majority of subsequently diagnosed SH within this cohort. In our hands the lowest cut-off cortisol value which produced a specificity $>99\%$ was 109 nmol/L. However, this was associated with lower sensitivity (56%) and hence performed poorly as a screening test. As anticipated, the ONDST did not differentiate between ACTH-dependent and independent forms of hypercortisolaemia. Accordingly, we support the traditional cortisol threshold of ≥ 1.8 mcg/dL (≥ 50 nmol/L) in the ONDST as the trigger for further confirmatory testing as recommended in the Endocrine Society and European Society guidelines (4).

The utility of urinary free cortisol in the diagnosis of SH and in the broader context of Cushing's syndrome has long been debated. There are numerous pitfalls associated with this test. Urine collection over a 24 h period is not always convenient for the patient and it is often difficult to obtain a complete sample in the clinic setting. Several studies have shown that 24 h UFC measurement performs poorly overall both as a screening and a diagnostic test, particularly if reliance is placed on a single measurement (20). Within our cohort 24 h UFC (measured on two occasions with the highest value used for subsequent analyses) demonstrated a low sensitivity (67%) and specificity (69%), although values ≥ 2 times the upper limit of the reference range carried a specificity $>99\%$ but a sensitivity of only 25% (Fig. 3). It is important to note however, that we used a univariate UFC measurement, rather than the multi-analyte, steroid metabolomic profiling that is increasingly available, with more recent

data suggesting superior sensitivity and specificity of urinary steroid metabolomics for the differential diagnosis of steroid excess in the context of adrenal disease (35).

In the setting of SH, ACTH levels are typically suppressed and we also observed this, with all affected patients returning levels <10 ng/L (<2.2 pmol/L). However, measurement of ACTH in the clinical setting carries multiple sampling challenges and can produce false positive results when sampled under non-ideal circumstances, reflecting the short half-life, pulsatile secretion and diurnal variability of ACTH release (4).

Measurement of DHEAS also offers some other potential advantages when screening in AI: firstly, it allows detection of tumors that are co-secretory (and therefore suspicious for malignancy); secondly, it has recently been recognized that some cases of congenital adrenal hyperplasia may present with AI, although we did not identify any such cases in our cohort (36).

It is important to note however that the reliability of low or suppressed DHEAS as a marker for SH test may be limited under certain circumstances, for example when there is pre-existing ACTH-suppression in the context of chronic glucocorticoid or opioid use, pituitary disease or hypothalamic dysfunction. Under these circumstances a false positive result may be obtained (37). However, each of the existing screening tests have well recognized limitations and pitfalls, and also require specific sampling conditions.

Our study has other potential limitations. The reported data are representative of a patient population attending a single center, using a single platform for DHEAS measurement. Multi-center studies and/or meta-analyses, specifically investigating the use of the DHEAS ratio reported here as a screening test for SH in the context of AI will be required to support and validate our findings, across patient populations, using different DHEAS assays. It will also be important to validate the utility of DHEAS ratio alone or in combination with the ONDST (at differing threshold values) for confirming or excluding SH without the need to progress to more expensive, time-consuming investigations.

299 Finally, low DHEAS has also been directly linked with increased cardiovascular risk (38), and it is
300 interesting to speculate that undiagnosed SH may underlie this association given the recent
301 demonstration that SH is associated with excess cardiovascular mortality (7,10,39).

302 In conclusion, our data support the use of an age-adjusted DHEAS ratio as a sensitive and specific
303 screening test for SH in patients with incidentally detected adrenal incidentalomas. Pending further
304 studies, we suggest that the identification of a low or suppressed DHEAS level in a patient with AI
305 should trigger more detailed assessment for SH.

306 **Acknowledgements**

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308 AKA, OK, ASP, DJH and MG are supported by the National Institute for Health Research Cambridge

309 Biomedical Research Centre.

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